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AcmA of *Lactococcus lactis* is an *N*-acetylglucosaminidase with an optimal number of LysM domains for proper functioning

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Keywords

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AcmA, the major autolysin of *Lactococcus lactis* MG1363 is a modular protein consisting of an N-terminal active site domain and a C-terminal peptidoglycan-binding domain. The active site domain is homologous to that of muramidase-2 of *Enterococcus hirae*, however, RP-HPLC analysis of mucopeptides released from *Bacillus subtilis* peptidoglycan, after digestion with AcmA, shows that AcmA is an *N*-acetylglucosaminidase. In the C-terminus of AcmA three highly similar repeated regions of 45 amino acid residues are present, which are separated by short nonhomologous sequences. The repeats of AcmA, which belong to the lysine motif (LysM) domain family, were consecutively deleted, removed, or, alternatively, one additional repeat was added, without destroying the cell wall-hydrolyzing activity of the enzyme *in vitro*, although AcmA activity was reduced in all cases. *In vivo*, proteins containing no or only one repeat did not give rise to autolysis of lactococcal cells, whereas separation of the producer cells from the chains was incomplete. Exogenously added AcmA deletion derivatives carrying two repeats or four repeats bound to lactococcal cells, whereas the derivative with no or one repeat did not. In conclusion, these results show that AcmA needs three LysM domains for optimal peptidoglycan binding and biological functioning.

In order to be able to grow and divide, bacteria produce several types of enzymes that can hydrolyze bonds in the peptidoglycan of the cell wall [1]. Two types of enzymes known as glycosidases hydrolyze the $\beta(1,4)$ bonds between the alternating *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the glycan chains in peptidoglycan. A lysozyme-like enzyme, β -*N*-acetylmuramidase (muramidase), hydrolyses *N*-acetylmuramyl,1,4- β -*N*-acetylglucosamine bonds, whereas the other, a β -*N*-acetylglucosaminidase (glucosaminidase), liberates free reducing groups of *N*-acetylglucosamine. In addition to these glycosidases, bacteria produce amidases, hydrolyzing the bond between the

glycan chain and the peptide side chain, and peptidases of varying specificity.

AcmA is the major autolysin of the Gram-positive bacterium *Lactococcus lactis* ssp. *cremoris* MG1363. AcmA is required for cell separation and is responsible for lysis in the stationary phase [2,3]. The 40.3 kDa secreted mature AcmA is subject to proteolytic degradation resulting in a number of activity bands in a zymogram of the supernatant of a lactococcal culture [4,5]. Bands as small as that corresponding to a protein of 29 kDa have been detected [3]. As no activity bands are produced by an *L. lactis* *acmA* deletion mutant, all bands represent products of AcmA [3]. Poquet *et al.*

Abbreviations

IPTG, isopropyl thio- β -D-galactoside; LysM, lysin motif; PepX, X-prolyl dipeptidyl aminopeptidase; X-gal, 5-bromo-4-chloroindol-3-yl- β -D-galactoside.

[5] have shown that the major surface housekeeping protease of *L. lactis* IL1403, HtrA, is capable of degrading AcmA. No AcmA degradation products were found in an *htrA* knockout mutant, in which HtrA is not expressed.

AcmA consists of an active site domain, followed by a C-terminal region (cA) containing three highly homologous repeats of 45 amino acids each, also called lysin motif (LysM) domains [3,6]. The active site domain is homologous to that of muramidase-2 of *Enterococcus faecalis*, suggesting that AcmA is also a muramidase [3]. However, the AcmA homologs AcmB [7], AcmC [8] and LytG [9] have been shown to be glucosaminidases. Amino acid substitutions in the AcmA homolog FlgJ of *Salmonella typhimurium* have shown that two conserved amino acid residues, aspartyl and glutamyl, which are also preserved in AcmA, muramidase-2 and LytG, are part of the putative active center of this peptidoglycan hydrolase that is essential for flagellar rod formation [10]. In the sequence of the genome of *L. lactis* IL1403 genes putatively encoding cell wall hydrolases with an active site homologous to that of AcmA are present (*acmB*, *acmC* and *acmD*). AcmD, like AcmA, contains three LysM domains, AcmB contains another cell-wall-binding motif, whereas AcmC does not contain a cell-wall-binding motif [11].

The C-terminal LysM domains of AcmA are involved in cell-wall binding [12]. Localization studies with the repeats have shown that the protein binds the cell surface of Gram-positive bacteria in a highly localized manner. The protein binds mainly at and around the poles of *L. lactis* and *Lactobacillus casei*. A derivative of AcmA lacking all three LysM domains did not bind to cells [13].

The repeats in cA are called LysM domains, because they were originally identified in bacterial lysins [6]. Cell wall hydrolases of various bacterial species and of bacteriophages contain repeats similar to those present in AcmA. LysM domains are also present in bacterial virulence factors and in a number of eukaryotic proteins, but not in archaeal proteins [14]. From an analysis of proteins containing LysM domains it is clear that the number of domains and their position in the proteins differs greatly [14]. Many proteins contain only one LysM domain, for example, the prophage amidase XlyA of *Bacillus subtilis* [15]. Examples of proteins with more than one LysM domain are the cell wall-bound γ -D-glutamate-meso-diaminopimelate mureopeptidases LytE and LytF of *B. subtilis* (respectively three and five repeats in their N-termini) [16–19] and muramidase-2, a homolog of AcmA produced by *Enterococcus hirae* (six LysM domains) [20].

The aim of this study was to investigate the modular structure of AcmA. This was done by consecutively deleting or adding C-terminal LysM domains. Furthermore, the specificity of the active site domain was investigated using RP-HPLC analysis of muropeptides released by AcmA from peptidoglycan. Although AcmA is highly homologous to muramidase-2, we show that AcmA is an *N*-acetylglucosaminidase.

Results

Two of the three repeats in AcmA are sufficient for cell separation and autolysis of cells

Several mutant AcmA derivatives were constructed to investigate the function of the three LysM domains in the C-terminus of AcmA. Because expression of AcmA in *Escherichia coli* results in growth problems followed by severe lysis [3], cloning and expression were performed in *L. lactis* MG1363. A stop codon was introduced behind the codon for Thr287 (pGKAL4) or Ser363 (pGKAL3) (Fig. 1B). Plasmid pGKAL4-specified AcmA (A1) contains only the first (most N-terminal) of the three repeats, whereas pGKAL3 specifies an AcmA variant (A2) carrying the first two repeats. pGKAL5 encodes an AcmA derivative lacking all repeats (A0) due to the introduction of a stop codon after Ser218. AcmA specified by pGKAL6 contains one and a half repeats (A1.5) owing to the presence of a stop codon behind the Ser339 codon. From pGKAL7 an AcmA mutant (A4) is produced that carries an additional (fourth) repeat as the result of a duplication of the polypeptide from Ser263 to Thr338. All proteins were expressed from the *acmA* promoter in the AcmA-negative strain *L. lactis* MG1363*acmAΔ1*. The various deletion derivatives of AcmA were examined with respect to the following properties: (a) their effect on halo formation on plates containing cell wall fragments of *Micrococcus lysodeikticus*; (b) the chain length of the cells expressing the mutant enzymes, and sedimentation of the cells in standing cultures; (c) their enzymatic activities, both in the cell and supernatant fractions; and (d) autolysis of producer cells.

Halo formation

On a G¹/₂M17 plate containing cell wall fragments of *M. lysodeikticus*, halos were absent when MG1363-*acmAΔ1* carried pGK13 or pGKAL5. All other strains produced a clear halo that differed in size. Halo size was correlated with the number of full-length repeats,

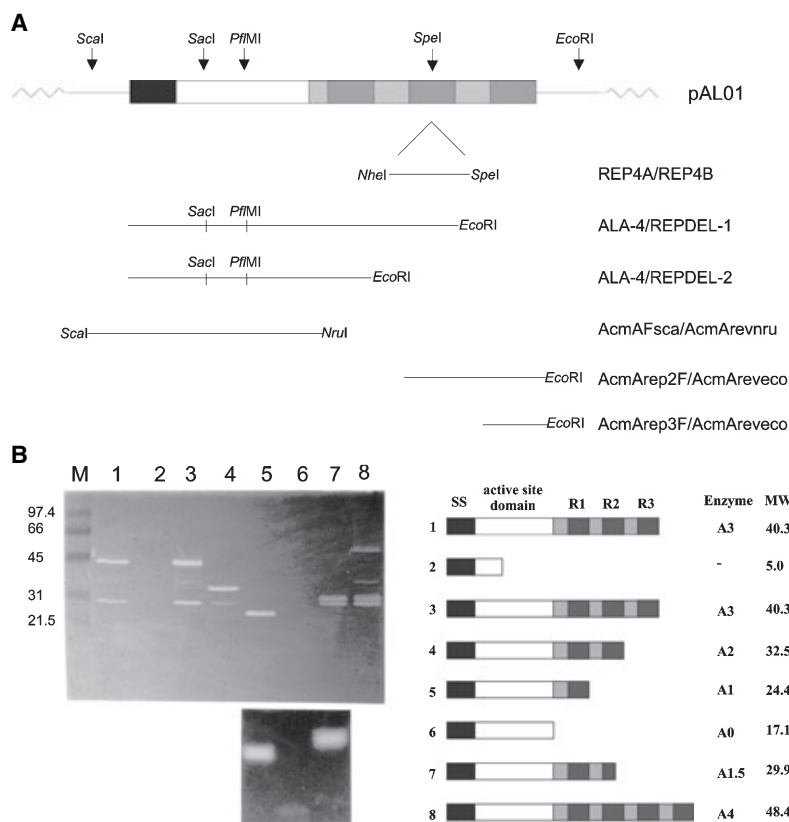


Fig. 1. (A) Detail of plasmid pAL01. Black box, signal sequence of AcmA; gray boxes, LysM domains; light gray boxes, linker regions preceding LysM domains. Restriction sites used to construct AcmA derivatives are depicted. PCR products REP4A/REP4B, ALA-4/REPDEL-1, ALA-4/REPDEL-2, AcmAFsca/AcmArevnru, AcmArep2F/AcmAreveco and AcmArep3F/AcmAreveco that were used to construct plasmids expressing AcmA derivatives A4, A2, A1, A2(R2 & 3) and A1(R3), respectively, are indicated by lines. (B) Lane 1: zymographic analysis of AcmA activity in supernatant fractions of end-exponential phase culture of MG1363 containing pGK13. Lanes 2–8: *L. lactis* MG1363acmA41 containing either pGK13, not encoding AcmA (2), pGKAL1, encoding enzyme A3 (3), pGKAL3, encoding enzyme A2 (4), pGKAL4, encoding enzyme A1 (5), pGKAL5, encoding enzyme A0 (6), pGKAL6, encoding enzyme A1.5 (7), or pGKAL7, encoding enzyme A4 (8). Cell extracts and supernatant samples were separated in an SDS/(12.5%) PAA gel containing 0.15% *M. lysodeikticus* autoclaved cells, and the proteins were subsequently renatured by washing the gel in a buffer containing Triton X-100. AcmA activity is visible as clearing zones in the gel. Molecular masses (kDa) of standard proteins (lane M) are shown in the left margin. Below the gel the lower part of lanes 5, 6 and 7 of the same gel is shown after 1 week of renaturation. The right half of the figure gives a schematic representation of the various AcmA derivatives. SS (black), signal sequence; Rx (dark gray), repeats; light gray, Thr, Ser and Asn-rich intervening sequences [3]; arrows, artificially duplicated region in the AcmA derivative containing four repeats. The active site domain is shown in white. MW, expected molecular sizes in kDa of the secreted forms of the AcmA derivatives. The numbers of the AcmA derivatives correspond with the lane numbers above the gel.

although the addition of an extra repeat resulted in a reduced halo size (Table 1). Apparently exactly three repeats are required for optimal cell wall lytic activity of AcmA.

Cell separation and sedimentation

Deletion of two and all three repeats had a clear effect on the chain length and sedimentation of the cells after growth overnight (Table 1). Thus, efficient cell separation requires at least two repeats in AcmA.

Enzyme activity

Cells and supernatants of overnight cultures of all strains were analyzed for AcmA activity by SDS/PAGE. No activity was detected in the cell fractions of cells expressing A0, even after 1 week of renaturation of the protein (Table 1). Of the other derivatives, two major activity bands were present in the cell fraction. In each case, their positions corresponded to proteins with the calculated molecular masses of the unprocessed and processed forms. As shown in

Table 1. Properties of *L. lactis*-expressing AcmA derivatives. The different strains were investigated for cellular lysis caused by the AcmA derivatives, by measuring the percentage of reduction in D_{600} of the cultures and by measuring the activity of the intracellular enzyme PepX released into the culture supernatants, 60 h after reaching the maximum D_{600} . Chain length, halo size surrounding colonies on plates containing *M. lysodeikticus* cells, sedimentation of the cells, AcmA activity in cell extracts and supernatants and cell binding properties were also investigated. Sup, supernatant fraction; Cfe, cell-free extract.

Number ^a	Strain (plasmid) ^b	AcmA variant ^c	Cell lysis (%) reduction in D_{600} ^d	PepX activity in supernatant ^e	Chain length ^f	Halo size ^g	Sedimentation ^h	Acm activity ⁱ sup	Cfe	Cell binding ^j
1	MG pGK13	A3	32.6	16.9	A	3.1	–	+	+	+
2	$\Delta 1$ pGK13	–	15.2	0.3	C	0	+	–	–	–
3	$\Delta 1$ pGKAL1	A3	36.7	19.8	A	5.0	–	+	+	+
4	$\Delta 1$ pGKAL3	A2	29.3	13.3	A	4.6	–	+	+	+
5	$\Delta 1$ pGKAL4	A1	18.8	0.4	B	3.9	+	+	+	+
6	$\Delta 1$ pGKAL5	A0	15.6	0.3	C	0	+	+	–	–
7	$\Delta 1$ pGKAL6	A1.5	18.6	1.6	B	2.2	+/-	+	+	+
8	$\Delta 1$ pGKAL7	A4	21.1	4.9	A	4.0	–	+	+	+

^a Corresponds to the AcmA derivative produced (Fig. 1). ^b MG: *L. lactis* MG1363, $\Delta 1$: *L. lactis* MG1363acmA $\Delta 1$. ^c –, no AcmA produced; Ax, AcmA with x repeats. ^d The reduction in D_{600} was calculated using $[(D_{\max} - D_{60\text{ h}})/D_{\max}] \times 100\%$. ^e Activity is in arbitrary units measured as the increase in D_{405} over time. ^f End exponential phase $\frac{1}{2}$ GM17 cultures were subjected to light microscopic analysis. A, mainly single cells and some chains up to five cells in length; B, some single cells but mainly chains longer than five cells; C, no single cells, only very long chains. ^g Halo size was measured in mm from the border of the colony after 45 h of incubation at 30 °C. ^h Analyzed by visual inspection of standing $\frac{1}{2}$ GM17 cultures after overnight growth in test tubes. ⁱ In zymograms of samples from end-exponential phase $\frac{1}{2}$ GM17 cultures. ^j Binding of AcmA derivatives in supernatants of end-exponential phase $\frac{1}{2}$ GM17 cultures to end-exponential phase cells of *L. lactis* MG1363acmA $\Delta 1$ after 20 min of incubation at 30 °C (see text for details).

Fig. 1B, all AcmA derivatives, except A0, were still active in the supernatant fractions. AcmA produced the characteristic breakdown pattern as determined previously [3]. All AcmA derivatives except A0 and A1 showed a distinct and highly reproducible degradation pattern. Two additional breakdown products were visible in the A4 and A1.5 preparation after prolonged renaturation (results not shown). Upon prolonged incubation of the zymogram, AcmA derivative A2 also showed this double band (result not shown). The cleavage sites in the C-terminal domain of AcmA that are responsible for this breakdown product are likely to be more easily accessible in the derivatives with 1.5 and 4 repeats. These data indicate that removal of the repeats does not destroy AcmA activity on *M. lysodeikticus* cell walls *in vitro*.

Autolysis

To analyze the effect of the repeats on autolysis of *L. lactis* during the stationary phase, overnight cultures of all strains were diluted 100-fold in $G^{1/2}$ M17 and incubated at 30 °C for 6 days while following the decrease in attenuation (D_{600}). All cultures exhibited similar growth rates, reached the same maximal absorbance and did not lyse during the exponential phase of growth. After ≈ 60 h of incubation maximal reduction in D_{600} was reached in all cases. The results are presented in Table 1 and show that auto-

lysis is optimal when three LysM domains are present. Deletion or addition of LysM domains results in reduced lysis. To investigate whether the decrease in D_{600} really reflected autolysis, the activity of the intracellular enzyme X-prolyl dipeptidyl aminopeptidase (PepX) was measured. After 60 h of incubation, PepX activity in the culture medium was also maximal in all samples, decreasing in all cases upon further incubation. Even though a considerable reduction in absorbance was obtained, hardly any PepX activity was detected in the supernatant of *L. lactis* MG1363acmA $\Delta 1$ and in cultures producing A0, A1 or A1.5. The reduction in absorbance might be due to cell morphological and/or intracellular changes influencing light scattering [2] or to activity of the other cell wall hydrolases not resulting in cell lysis. In contrast, a considerable quantity of PepX was released into the supernatant of cultures producing A2 and A3. Thus, two repeats in AcmA are sufficient for autolysis of *L. lactis*. A2 and A4 production led to reduced lysis of producer cells. PepX was released from MG1363acmA $\Delta 1$ cells only when they were incubated in supernatants of cultures producing the AcmA derivatives A3 or A4. At least three repeats should therefore be present to obtain lysis *in trans* (results not shown). Taken together, these results indicate that the repeats in AcmA determine the efficiency of cell autolysis and are required for cell separation.

Binding properties of the AcmA derivatives

To investigate whether the differences in autolysis and separation of cells expressing the various AcmA derivatives are caused by differences in cell-wall binding of the AcmA mutants, direct binding studies were performed. Antibodies were raised against the active site of AcmA to be able western hybridization studies. The active site in the N-terminal domain of AcmA (amino acids 58 to 218 of AcmA) was fused to the thioredoxin using plasmid pET32A. As the fusion protein, which comprises 326 amino acids, does not have cell wall hydrolase activity (Fig. 2B) overexpression in *E. coli* was successful. A protein with the expected molecular mass (35 kDa) was isolated from a culture of *E. coli* BL21(DE3) (pETAcmA) (Fig. 2). By cleavage with enterokinase, the protein was split into a thioredoxin part of 17 kDa and an AcmA domain of 18 kDa. The 18 kDa AcmA active site was active after prolonged incubation, as shown on a zymogram containing *M. lysodeikticus* autoclaved cells (Fig. 2B). The AcmA domain was subsequently used to raise anti-AcMA IgG in rabbits.

As shown in Fig. 1, AcmA is subject to degradation when expressed in *L. lactis* MG1363*acmAΔ1*. HtrA, a cell surface protease from *L. lactis*, is responsible for

the specific degradation of AcmA [5]. An *htrA* mutant of *L. lactis* NZ9000 *acmA* was therefore used to produce the AcmA derivatives and to analyze their binding in the absence of a background of degradation products. The supernatant of *L. lactis* NZ9000 *acmAΔ1 ΔhtrA* carrying pGKAL1, pGKAL3, pGKAL4 or pGKAL7 was analyzed for AcmA activity. As shown in Fig. 3A, breakdown products of AcmA are indeed absent when the enzyme was expressed in this double mutant. Halo formation, cell sedimentation, autolysis and cell separation were comparable with the equivalent MG1363*acmAΔ1* strain (results not shown).

Binding of the AcmA derivatives to cells was subsequently studied using anti-AcMA IgG. Equal amounts of MG1363*acmAΔ1* cells were resuspended and incubated in 1 mL of supernatants of *L. lactis* NZ9000 *acmAΔ1 ΔhtrA* cultures containing the various AcmA derivatives. The suspensions were centrifuged and the cell pellet (cell-bound AcmA) and the supernatant (nonbound AcmA) analyzed for the presence of AcmA by western hybridization. Binding was only observed for AcmA derivatives A4, A3 and A2 (Fig. 3B). Of these three, A2 and A4 bound much more weakly to the cells than did A3, the wild-type enzyme. The results are consistent with the lysis results (Table 1).

Enzyme A1 does not bind to lactococcal cells. This can be explained in two ways: first, the LysM domain is not sufficient to bind AcmA to cells, or this LysM domain is not functional. Furthermore, enzyme A2 binds more weakly to cells than enzyme A3, which may be because LysM domain 3 is the best binding LysM domain of AcmA. Removing LysM 3 would, therefore, result in decreased binding of AcmA. To address this, two additional derivatives of AcmA were constructed. In variant A2(R2 & 3), the region containing LysM domains 2 and 3 was fused directly downstream of the linker region that connects the active site domain and the first LysM domain in wild-type AcmA. In variant A1(R3) only the third LysM domain was fused to that region. The new AcmA variants were expressed in *L. lactis* MG1363*acmAΔ1* and cell fractions and supernatant samples were analyzed on a zymogram. A2(R2 & 3) and A1(R3) were both active and no differences were observed when compared to cell fractions and supernatants of enzymes A2 and A1 (results not shown). Cell lysis upon expression of the two new AcmA variants was compared with lysis by variants A2 and A1 by measuring the amounts of PepX released after 48 h. Approximately the same amounts PepX were released upon expression of A2 and A2(R2 & 3) (Fig. 4A). Expression of variant A1(R3) resulted in very low amounts of PepX released, as is the case with

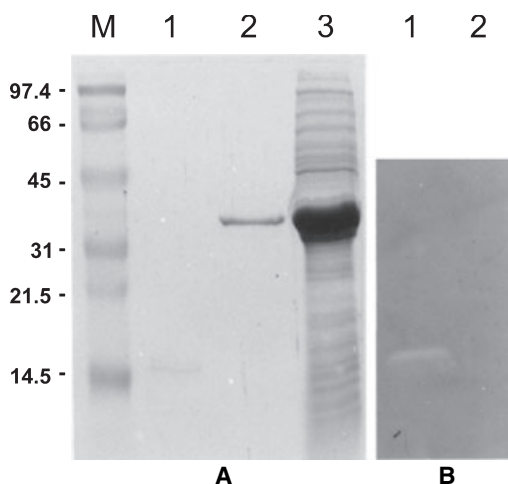
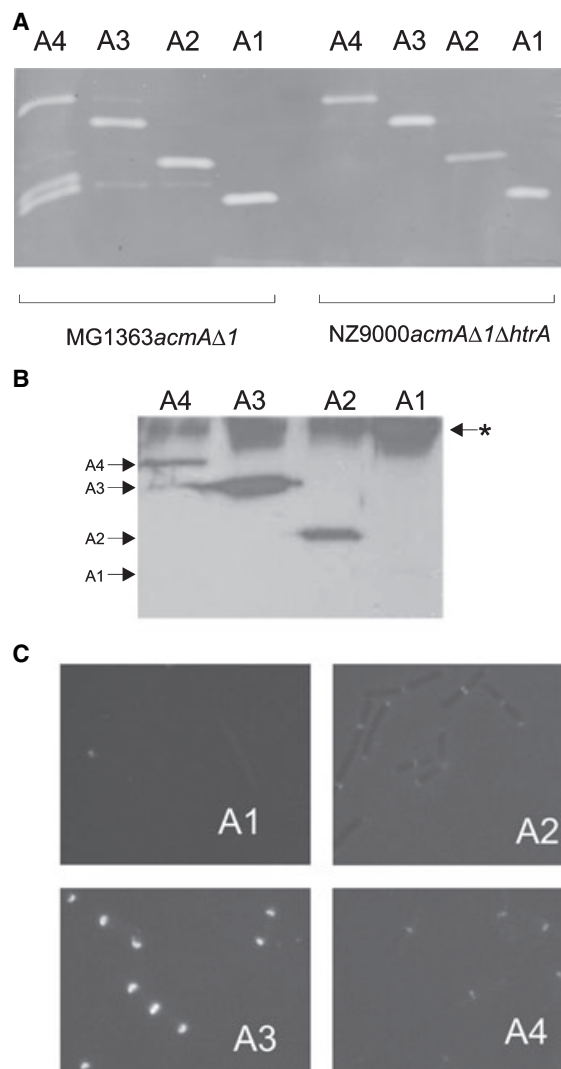


Fig. 2. Overexpression and purification of the active-site domain of AcmA. (A) SDS/12.5% PAGE of cell extracts of 10 μ L of *E. coli* BL21(DE3) (pETAcmA) (lane 3) induced for 4 h with IPTG. Lane 2: 10 μ L of purified fusion protein isolated from 25 μ L of induced *E. coli* culture. Lane 1: 10 μ L of the enterokinase cleaved protein. (B) Renaturing SDS/12.5% PAGE with 0.15% *M. lysodeikticus* autoclaved cells using the same amount of the samples 1 and 2 shown in (A). Molecular masses (kDa) of standard proteins are shown on the left of the gel. Before loading, the samples were mixed with an equal volume of 2 \times sample buffer [36].



AcMA variant A1. Cell binding of A2(R2 & 3) was compared with binding of A2: same amounts were able to bind to cells (Fig. 4B). A1(R3) did not bind to cells, and therefore behaves like enzyme A1.

Localization of AcmA and its derivatives on the cell surface

Using the anti-AcMA IgG and immunofluorescence microscopy, the AcMA derivatives used in this study were examined for their ability to bind to bacterial cell surfaces when added from the outside. Binding of AcMA on the lactococcal surface was very inefficient and fluorescence was hardly detectable (results not shown). The AcMA derivatives A2, A3 and A4 could be detected on the cell surface of *Lb. casei* (Fig. 3C). AcMA binding is highly localized at the poles of these

Fig. 3. (A) Expression of AcMA derivatives A1, A2, A3 and A4 in the *L. lactis* NZ9000 mutants *acmAΔ1* and *acmAΔ1 ΔhtrA*, visualized by zymographic analysis of culture supernatants of cells expressing the AcMA variants. (B) Binding of the AcMA derivatives A1, A2, A3 and A4 to *L. lactis* cells. Stationary phase cells from 1 mL of *L. lactis* MG1363*acmAΔ1* culture were mixed with the supernatant of stationary phase cultures of *L. lactis* NZ9000*acmAΔ1*, *ΔhtrA* expressing A1, A2, A3 or A4. After allowing 5 min of binding, cells were collected by centrifugation. Proteins bound to cells were separated by SDS/12.5% PAGE and blotted onto poly(vinylidene difluoride) membranes. AcMA antigen was visualized using the AcMA-specific polyclonal antibodies and subsequent chemoluminescence detection. The asterisk indicates *L. lactis* protein that reacts with the AcMA antibodies due to an impurity in the antibody preparation (data not shown). (C) Localization of AcMA and its derivatives on the cell surface of *Lb. casei*. Cells of overnight cultures of *Lb. casei* were mixed with supernatant of *L. lactis* NZ9000*acmAΔ1*, *ΔhtrA* containing A1, A2, A3 or A4 protein. Surface bound protein was subsequently detected by immunofluorescence microscopy using anti-(AcMA rabbit) polyclonal IgG and anti-rabbit IgG conjugated with the fluorescent probe Oregon Green (Molecular Probes). Bound AcMA protein is visible as bright green patches on the cell surface.

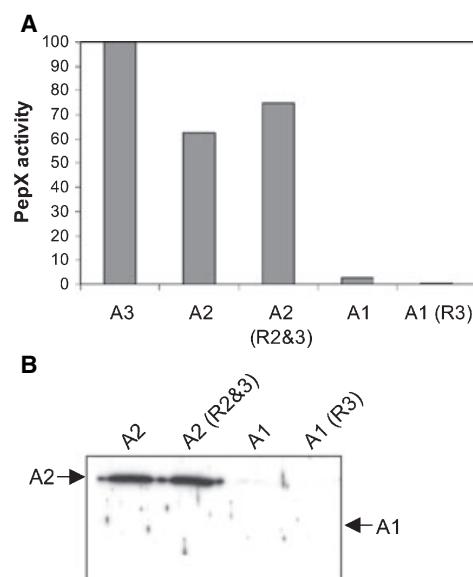


Fig. 4. (A) PepX release upon expression of AcMA derivatives A3, A2, A2(R2 & 3), A1 and A1 (R3). *L. lactis* MG1363*acmAΔ1* expressing the AcMA derivatives were grown for 48 h and subsequently the amount of PepX present in the supernatant was determined. The amount of PepX released by expression of A3 was set to 100%. The results shown are the averages of two parallel experiments. (B) Binding of AcMA derivatives A2, A2(R2 & 3), A1 and A1(R3) to *L. lactis* MG1363*acmAΔ1*. The experiment was performed as described in the legend to Fig. 3B.

cells. Binding of A2 and A4 was less efficient than binding of A3, as evidenced by the lower fluorescence intensity.

Isolation of mature AcmA and determination of its specificity

The N-terminus of AcmA is homologous to several other peptidoglycan hydrolases, among which are muramidase-2 of *Ent. hirae* and FlgJ of *S. typhimurium*. Based on this homology and early biochemical data and lactococcal autolysins, AcmA has been named a muramidase [3]. Its hydrolytic activity, however, has not been studied thoroughly. To be able to investigate the activity of AcmA, the enzyme was concentrated by making use of its peptidoglycan-binding properties. *L. lactis* MG1363*acmAΔ1* cells were treated with 10% (w/v) SDS and 10% (v/v) TCA to increase their AcmA binding capacity [13] and were subsequently mixed with the supernatant of an *L. lactis* MG1363 culture. The suspension was pelleted and the

peptidoglycan-bound proteins were extracted using 8 M LiCl. After dialysis, AcmA activity could be detected as a decrease in A_{600} when the extract was mixed with autoclaved cells of *M. lysodeicticus* (results not shown). Peptidoglycan binding proteins isolated in the same way from the supernatant of an *L. lactis* MG1363-*acmAΔ1* culture did not show lytic activity (results not shown).

AcmA is active against peptidoglycans of different structural types including that of *B. subtilis*. *B. subtilis* peptidoglycan was hydrolyzed with the partially purified, concentrated AcmA preparation. The mixture was centrifuged, after which the supernatant [containing all the soluble (released) peptidoglycan fragments] was reduced with borohydride and resolved using RP-HPLC. The chromatogram shows two major peaks, indicated with arrows in Fig. 5A. No peaks were

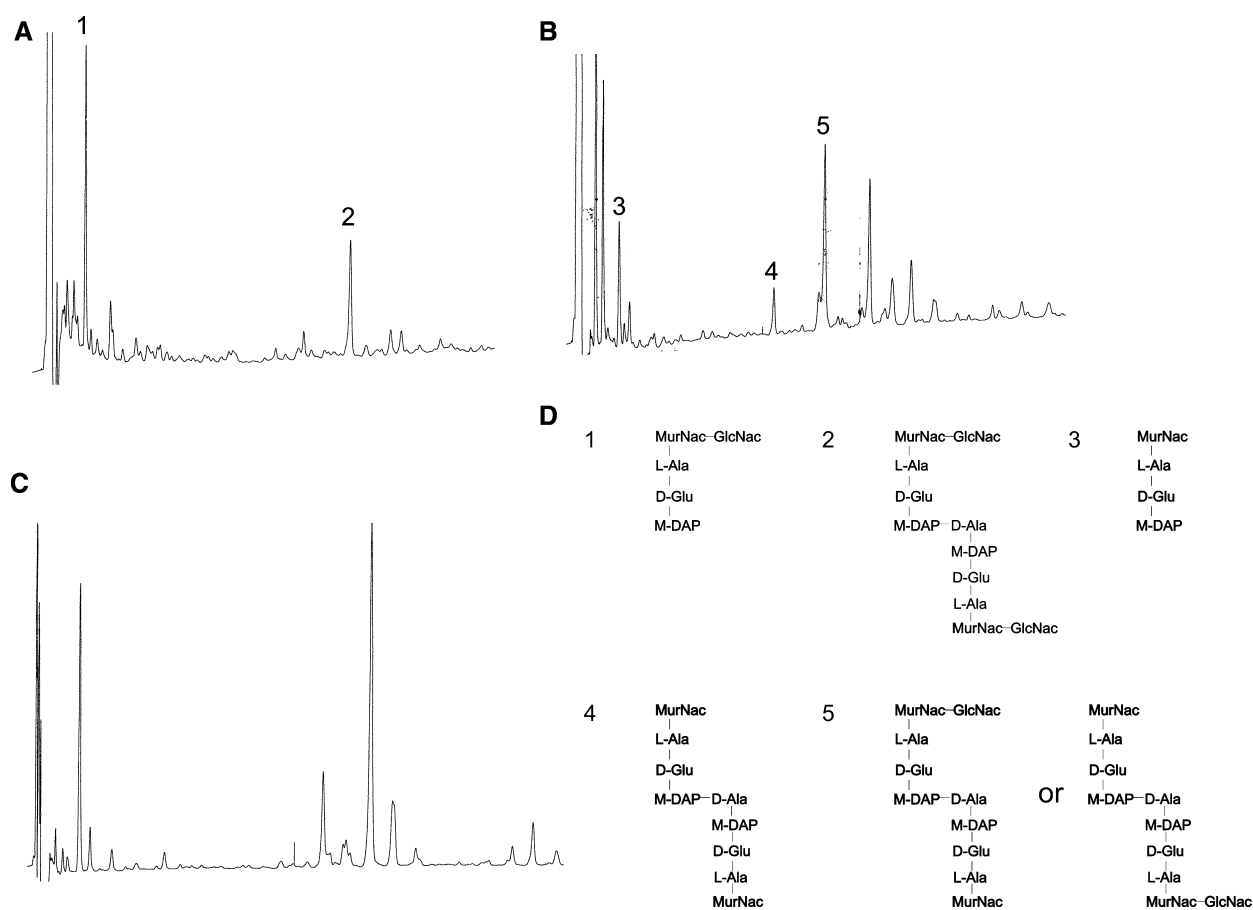


Fig. 5. Identification of the hydrolytic specificity of AcmA by RP-HPLC of mucopeptides. (A) RP-HPLC elution pattern of mucopeptides released by AcmA from *B. subtilis* peptidoglycan. Purified AcmA-digested peptidoglycan samples were separated on an octadecylsilane column, and the A_{202} of the eluate was monitored. Arrows indicate the two major AcmA-specific peaks in the eluate. (B) RP-HPLC chromatogram of Cellosyl digested mucopeptides that were released from *B. subtilis* peptidoglycan by AcmA. *B. subtilis* peptidoglycan was incubated with AcmA, the soluble peptidoglycan fragments were subsequently incubated with Cellosyl and reduced with borohydride. (C) RP-HPLC chromatogram of mucopeptides released from *B. subtilis* peptidoglycan by Cellosyl. (D) Structure of glucosaminidase-specific mucopeptides [9,21]. Numbers refer to peaks in Fig. 4A,B,C.

observed in the chromatogram of peptidoglycan treated with the peptidoglycan-binding protein preparation of the supernatant of *L. lactis* MG1363*acmAΔ1*, which does not express AcmA (results not shown). These peaks were compared with those obtained by hydrolysis of *Bacillus* peptidoglycan with the muramidase Cellosyl (Fig. 5C) [21]. The AcmA-specific peaks were not identical to the major muramidase peaks.

To investigate whether the mucopeptides released from peptidoglycan by AcmA could be hydrolyzed by Cellosyl, they were incubated with Cellosyl and subjected to RP-HPLC analysis. The AcmA-specific peaks disappeared and new peaks appeared in the trace (Fig. 5B). Because the mucopeptides released by AcmA could apparently be hydrolyzed by a true muramidase, AcmA is not a muramidase.

To examine whether the AcmA-specific mucopeptides are products of glucosaminidase activity, the HPLC traces were spiked with mucopeptides obtained by hydrolysis of peptidoglycan with the *B. subtilis* glucosaminidase LytG, a homolog of AcmA [9]. These LytG-specific mucopeptides were analyzed by RP-HPLC like the AcmA-specific mucopeptides. The structures of the LytG mucopeptides were determined using NMR [9]. The retention times of mucopeptides released from the peptidoglycan by AcmA were identical to those of the mucopeptides released by LytG (results not shown), identifying AcmA as an *N*-acetylglucosaminidase. The structures of the glucosaminidase-specific mucopeptides (numbered peaks in Fig. 5A,B) are given in Fig. 5D. AcmA releases mucopeptides with *N*-acetylglucosamine at the reducing terminus (mucopeptides 1 and 2 in Fig. 4D). These *N*-acetylglucosamines can be substrates for Cellosyl, resulting in mucopeptide-3, -4 and -5. The trace of the small soluble peptidoglycan fragments generated by the incubation of isolated peptidoglycan with Cellosyl did not change after incubation of these fragments with partially purified AcmA (results not shown), suggesting that small mucopeptides are not substrates for AcmA.

Discussion

We studied the modular organization of AcmA, an enzyme consisting of two separate domains [3]. The overproduced and purified N-terminal region, from amino acid residue 58 to 218 in the preprotein, is active on *M. lysodeicticus* cell walls and, thus, contains the active site of the enzyme. This is in agreement with the finding that the repeatless AcmA mutant A0 can still hydrolyze *M. lysodeicticus* cell walls, albeit with severely reduced efficiency [13]. Prolonged renaturation was needed to detect the activity of the enzyme *in vitro*, whereas

colonies producing the protein did not form a halo on plates containing *M. lysodeicticus* cell walls.

The sequence of the N-terminal active site domain of AcmA is homologous to that of muramidase-2 of *Ent. hirae*. In this study we show, however, that AcmA is not a muramidase but a glucosaminidase. Various methods to determine the hydrolytic specificity of glycosidases have been published. Peptidoglycan fragments obtained after hydrolysis with muramidase-2 of *Ent. hirae* were reduced with radioactive borohydride. Samples were analyzed after complete acid hydrolysis by ion-exchange chromatography. As the single labeled product that was detected had the same behavior as standard reduced muramic acid, Mur2 was shown to be a muramidase [22]. Pesticin, a bacteriocin produced by *Yersinia pestis* has been shown to be a muramidase by analyzing the products released from peptidoglycan by RP-HPLC and comparing the products with those released by the muramidase lysozyme [23]. In the same study, the radioactive borohydride method was also used to confirm that pesticin is a muramidase.

The RP-HPLC analysis we used in this study to determine the specificity of AcmA relies on extensive knowledge of the mucopeptides released from the *B. subtilis* peptidoglycan [9,21]. From each peak in the chromatogram of a muramidase digest of the vegetative peptidoglycan the exact structure of the constituting mucopeptide is known. Using this method the AcmA homologs AcmB [7], AcmC [8] and LytG [9] were shown to have glucosaminidase activity. This method also proved to be a powerful tool in the analysis of AcmA specificity. AcmA is not capable of hydrolyzing small mucopeptides, in our case peptidoglycan fragments released by the muramidase Cellosyl from the *B. subtilis* peptidoglycan. This can be explained by the suggestion that AcmA is not able to bind small peptidoglycan parts, as binding is necessary for activity of AcmA. Also the active site domain of AcmA could be dependent on big peptidoglycan parts as a substrate.

The C-terminal domain of AcmA with the three LysM domains was analyzed by deleting and addition of LysM domains. Enzymes A1, A2 and A4 had *in vitro* activities, as determined in a zymogram, which were nearly the same as that of the wild-type protein, although in the plate assay A1 produced a smaller halo than A2, which, in turn, was smaller than that produced by the wild-type A3. Also, A4 produced a smaller halo than wild-type AcmA. Taken together, these results indicate that, although the N-terminus of AcmA contains the active site, the presence of at least one complete repeat is needed for the enzyme to retain appreciable activity *in vitro*, whereas optimal activity is obtained with three repeats. A similar result was

obtained for the active site domain of the FlgJ protein of *S. typhimurium*, a muramidase-like enzyme involved in flagellar rod formation [10]. The N-terminal half of FlgJ is dispensable for peptidoglycan-hydrolyzing activity *in vitro*, but the truncated protein does not support cellular flagellation.

A strain producing A1 grows in longer chains than a strain expressing A2 and, in contrast to A2-producing cells, sedimented and did not autolyse. Only cultures producing AcmA with two or more full-length repeats are subject to autolysis and produce chains of wild-type length. Binding studies, using antibodies raised against the active site domain of AcmA, with the AcmA derivatives supported the lysis and cell separation results. To prevent degradation of AcmA by HtrA, the AcmA derivatives were expressed in the HtrA-negative mutant NZ9000 (*acmAΔ1 ΔhtrA*). AcmA derivative A1 was not able to bind to cells when it was added from the outside. A2 and A4 were able to bind to cells, albeit with lower efficiencies. The highest efficiency was obtained when three repeats were present in AcmA, i.e. with the wild-type enzyme. Enzymes A1 and A1(R3) do not bind to cells, which shows that one repeat in AcmA is not enough for cell wall binding. The lower binding efficiency of variant A2 could suggest that the third LysM domain of AcmA is the most important for binding. However, enzyme A2(R2 & 3) binds with the same efficiency to cells as A2 and expression in *L. lactis* MG1363*acmAΔ1* results in approximately the same degree of lysis for both enzyme A2 as enzyme A2(R2 & 3). These results show that LysM domains 1 and 3 are equally functional, despite the amino acid differences. In conclusion, the number of LysM domains present in the protein determines the binding efficiency of the protein, with optimal binding when three LysM domains are present.

The results of the binding studies are in full agreement with the results on cell separation and autolysis: the number of repeats in AcmA affects the binding efficiency and, consequently, the *in vivo* activity of the enzyme. The *B. subtilis* glucosaminidase LytD has a duplication of two types of direct repeats in the N-terminus of the protein. Serial deletions from the N-terminus of the glucosaminidase revealed that the loss of more than one repeating unit drastically reduces the lytic activity of LytD toward cell walls [24]. The major pneumococcal LytA amidase has six repeating units in its C-terminus that recognize choline in (lipo)teichoic acids in the cell wall. Biochemical analyses of truncated LytA mutants showed that the amidase must contain at least four units to efficiently recognize the choline residues [25]. Loss of an additional unit dramatically reduces its hydrolytic activity as well as its binding capacity,

suggesting that the catalytic efficiency of LytA can be considerably improved by keeping the protein attached to the cell wall substrate.

A fusion protein consisting of the antigen MSA2 and the C-terminus of AcmA binds to specific locations on the cell surface of Gram-positive bacteria [13]. No AcmA could be detected by immunofluorescence on the cell surface of *L. lactis* MG1363*acmAΔ1* cells incubated with the AcmA deletion derivatives. Also, no AcmA is detectable on wild-type MG1363 cells or on *L. lactis* cells overproducing AcmA (results not shown). Apparently, the amount of AcmA present on the cell surface is not enough to allow detection with anti-AcmA IgG. Using more cells in western hybridization does show that AcmA binds to the cell surface. Deletion or addition of LysM domains altered only the binding efficiency of the AcmA derivatives, not the distribution on the cell surface of *Lb. casei*.

In a separate study [2], we showed that AcmA can operate intercellularly: AcmA-free lactococcal cells can be lysed when grown together with cells producing AcmA. Combining this observation with the results presented above allows us to conclude that AcmA not only binds when confronting a cell from the outside but, indeed, is capable of hydrolyzing the cell wall with concomitant lysis of the cell. A minimum of three repeats is needed for this to occur: derivative A2, containing two LysM domains is not able to lyse cells *in trans*, whereas derivative A4 is. Lysis does occur in cells expressing derivatives A2 and A4, although in this case A2 is more active than A4. This shows that the number of repeats in AcmA clearly affects the action of AcmA.

It is tempting to speculate that the apparent increase in catalytic activity concomitant with an increase in the number of repeats is caused by the repeat domains, allowing the enzyme to bind to its substrate, the peptidoglycan of the cell wall, more efficiently. As postulated by Knowles *et al.* [26] for the cellulose-binding domains in cellobiohydrolases, such binding would increase the local concentration of the enzyme. The repeats could be involved in binding alone or could be important for proper positioning of the catalytic domain towards its substrate. Moreover, it could allow 'scooting' of the enzyme along its polymeric substrate. The increase in AcmA activity with an increasing number of repeats to up to three in the wild-type enzyme, suggests an evolutionary process of repeat amplification to reach an optimum for proper enzyme functioning. The presence of five and six repeats in the very similar enzymes of *Ent. faecalis* and *Ent. hirae*, respectively, may reflect slight differences in cell wall structure and/or the catalytic domain, requiring the recruitment

by these autolysins of extra repeats for optimal enzyme activity. The number of LysM domains present in different proteins from the same organism is not necessarily constant. In the *B. subtilis* genome, genes encoding proteins with one (e.g. XlyA), two (e.g. YaaH), three (LytE), four (YojL) and five (LytF) LysM domains are found [15–19,27]. This suggests that for each protein the number of LysM domains is optimized.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2. *L. lactis* was grown at 30 °C in twofold diluted

M17 broth (Difco Laboratories, Detroit, MI) containing 0.5% glucose and 0.95% β -glycerophosphate (Sigma Chemical Co., St. Louis, MO) as standing cultures ($G^{1/2}$ M17). Agar plates of the same medium contained 1.5% agar. Five micrograms per milliliter of erythromycin (Roche, Mannheim, Germany) was added when needed. *E. coli* and *B. subtilis* were grown at 37 °C with vigorous agitation in TY medium (Difco), or on TY medium solidified with 1.5% agar. When required, the media contained 100 μ g of ampicillin (Sigma), 100 μ g erythromycin or 50 μ g kanamycin (both from Roche) per mL. *Lb. casei* was grown in MRS medium [28] at 37 °C.

Isopropyl thio- β -D-galactoside (IPTG) and 5-bromo-4-chloroindol-3-yl- β -D-galactoside (X-gal) were used at concentrations of 1 mM and 0.002%, respectively.

Table 2. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant phenotype(s) or genotype(s)	Reference
Strains		
<i>L. lactis</i> ssp. <i>cremoris</i>		
MG1363	Plasmid-free strain	[40]
MG1363 <i>acmA</i> Δ 1	Derivative of MG1363 carrying a 701-bp <i>SacI</i> / <i>SpeI</i> deletion in <i>acmA</i>	[3]
NZ9000 <i>acmA</i> Δ 1 Δ <i>htrA</i>	Derivative of NZ9000 carrying a 701-bp <i>SacI</i> / <i>SpeI</i> deletion in <i>acmA</i> , a deletion of <i>htrA</i> and a chromosomal insertion of <i>nisRK</i> in the <i>pepN</i> locus	[41]
<i>E. coli</i>		
NM522	<i>supE thi Δ(lac-proAB) Δhsd5(rK- mK-)[F' proAB lacIqZM15]</i>	Stratagene
BL21(DE3)	F [−] <i>ompT rB[−] mB- int</i> ; bacteriophage DE3 lysogen carrying the T7 RNA polymerase gene controlled by the lacUV5 promoter	[34]
Other strains		
<i>B. subtilis</i> 168	<i>trpC2</i>	[27]
<i>Lactobacillus casei</i> ATCC393	Type strain	ATCC
Plasmids		
pET32A	Ap ^r , vector for high level expression of thioredoxin fusion proteins	Novagen
pUK21	Km ^r , general cloning vector	[42]
pBluescript SK+	Ap ^r , general cloning vector	Stratagene
pAL01	Ap ^r , pUC19 carrying a 4137-bp lactococcal chromosomal DNA insert with <i>acmA</i> gene	[3]
pDEL1	Ap ^r , pBluescript SK+ with 785-bp <i>SacI</i> / <i>EcoRI</i> fragment of <i>acmA</i> obtained by PCR with primers ALA-4 and REPDEL-1	This study
pDEL2	Ap ^r , pBluescript SK+ with 554-bp <i>SacI</i> / <i>EcoRI</i> fragment of <i>acmA</i> obtained by PCR with primers ALA-4 and REPDEL-2	This study
pDEL3	Ap ^r , pBluescript SK+ with 348-bp <i>SacI</i> / <i>EcoRI</i> fragment of <i>acmA</i> obtained by PCR with primers ALA-4 and REPDEL-3	This study
pGKAL1	Em ^r , Cm ^r , pGK13 containing <i>acmA</i> under control of its own promoter on a 1942-bp <i>SspI</i> / <i>Bam</i> HI insert	[3]
pGKAL3	Em ^r , Cm ^r , pGKAL1 derivative expressing A2	This study
pGKAL4	Em ^r , Cm ^r , pGKAL1 derivative expressing A1	This study
pGKAL5	Em ^r , Cm ^r , pGKAL1 derivative expressing A0	[13]
pGKAL6	Em ^r , Cm ^r , pGKAL1 derivative expressing A1.5	This study
pGKAL7	Em ^r , Cm ^r , pGKAL1 derivative expressing A4	This study
pGKAL8	Em ^r , Cm ^r , pGKAL1 derivative expressing A2(R2 & 3)	This study
pGKAL9	Em ^r , Cm ^r , pGKAL1 derivative expressing A2(R3)	This study
pETAcmA	Ap ^r , pET32A expressing active site domain of <i>AcmA</i> from residues 58–218 fused to thioredoxin	This study

General DNA techniques and transformation

Molecular cloning techniques were performed essentially as described by Sambrook *et al.* [29]. Restriction enzymes, Klenow enzyme and T4 DNA ligase were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA) and were used according to the instructions of the supplier. Deoxynucleotides were obtained from Pharmacia LKB Biotechnology AB (Uppsala, Sweden) or BDH (Poole, UK). Electrotransformation of *E. coli* and *L. lactis* was performed using a gene pulser (Bio-Rad Laboratories, Richmond, CA), as described previously [30,31]. Plasmid DNA was isolated using the Qiagen plasmid DNA isolation kit (Qiagen GmbH, Hilden, Germany) or by CsCl-ethidiumbromide density gradient centrifugation. DNA fragments were isolated from agarose gels using the Qiagen gel extraction kit and protocols.

Primer synthesis, PCR and DNA sequencing

Synthetic oligo deoxyribonucleotides were synthesized with an Applied Biosystems 392 DNA/RNA synthesizer (Foster City, CA, USA). The sequences of the oligonucleotides are listed in Table 3.

PCR were performed in a Bio-Medical thermocycler 60 (Bio-Medical GmbH, Theres, Germany) using super *Taq* DNA polymerase and the instructions of the manufacturer (HT Biotechnology Ltd, Cambridge, UK). PCR fragments were purified using the nucleotide removal kit and protocols of Qiagen. Nucleotide sequences of double-stranded plasmid templates were determined using the dideoxy chain termination method [32] with the T7 sequencing kit and protocol (Pharmacia) or the automated fluorescent DNA sequencer 725 of Vistra Systems (Amersham Life Sciences, Little Chalfont, UK). Protein homology searches against the SWISSPROT, PIR, GenBank databases were carried out using the BLAST program [33].

Construction of *AcmA* derivatives

A stop codon and an *EcoRI* restriction enzyme site were introduced in *acmA* at the end of nucleotide sequences encoding repeat one and the same was done at the end of repeat two by PCR using the primers REPDEL-1 and REPDEL-2, with plasmid pAL01 as a template. Primer ALA-4, annealing within the sequence encoding the signal peptide of *AcmA*, was used in all cases as the upstream primer. Figure 1A gives details on the position of primers ALA-4 REPDEL-1 and REPDEL-2 and the restriction sites used for the construction of the *AcmA* derivatives. The two PCR products were digested with *SacI* and *EcoRI* and cloned into the corresponding sites of pBluescript SK+ leading to pDEL1 and pDEL2. Subsequently, the 1187-bp *PflMI*–*EcoRI* fragment of pGKAL1 (Fig. 1A) was replaced by the 513 and 282 bp *PflMI*–*EcoRI* fragments of the inserts of pDEL1 and pDEL2, respectively. The proper plasmids specifying proteins containing one or two repeats (pGKAL4 and pGKAL3, respectively) were obtained in *L. lactis* MG1363*acmAΔ1*. pGKAL1 was cut with *SpeI* (Fig. 1A). The sticky ends were flushed with Klenow enzyme and self-ligation introduced an UAG stop codon after the Ser339 of *acmA*. The resulting plasmid was named pGKAL6. A DNA fragment encoding half of the first repeat until the *SpeI* site in the middle of the second repeat was synthesized by PCR using primers REP4A and REP4B. The *NheI* and *SpeI* sites at the ends of the 250 bp PCR product were cut and the fragment was cloned into the unique *SpeI* site of pGKAL1, resulting in plasmid pGKAL7.

AcmA variants A2(R2 & 3) and A1(R3) were cloned as follows: the active site domain plus the linker region between the active site domain and the first LysM domain of *AcmA* was amplified using primers *AcmAFsca* (upstream of the *ScaI* site in pGKAL1; Fig. 1A) and *AcmArevnru*. The reverse primer inserted an *NruI* site in the linker region upstream of the first LysM domain, which

Table 3. Oligonucleotides used in this study. The indicated restriction enzyme (R/E) sites are underlined while stop codons are shown in italic.

Name	Nucleotide sequence (5'–3')	R/E site
REPDEL-1	CGCGAATTCAGATTATGAAACAATAAG	<i>EcoRI</i>
REPDEL-2	CGCGAATTC TTATGTCAGTACAAGTTTTTG	<i>EcoRI</i>
REPDEL-3	CGCGAATTC TTATGAAGAAGCTCCGTC	<i>EcoRI</i>
ALA-4	CTTCAACAGACAAGTCC	
REP4A	AGCAATACTAGTTTTATA	<i>SpeI</i>
REP4B	CGCGAATTCGCTAGCGTCGCTCAAATTCAAAGTGCG	<i>NheI</i>
ACMHIS	AGGAGATCTGCGACTAATCATCAGAGG	<i>BglII</i>
<i>AcmArevnru</i>	GCATGAATTCATCGCGAAGTCTATTGTTCAG	<i>NruI</i>
<i>AcmAFsca</i>	GGTACTGCCGGGCTCCTGCGG	
<i>AcmArep2F</i>	ACAACGTGTTAAGGTTAAATCCGGAGATACCCTTTGGGCG	
<i>AcmArep3F</i>	TCAATTCATAAGGTCGTTAAAGGAGATACTCTCTGG	
<i>AcmAreveco</i>	AGCGGAATTC AATAATTTATTTTATTCGTAGATACTGACC	<i>EcoRI</i>

resulted in a nonsense mutation of codon 242. The PCR product was subsequently digested with *Nru*I, which digests blunt directly behind codon 242. The region containing LysM domains 2 and 3, and the region containing LysM 3 were subsequently amplified using primers AcmArep2F and AcmArep3F, respectively, as forward primers and primer AcmAreveco as reverse primer. The resulting PCR products were directly ligated in the blunt *Nru*I site of the active site/linker PCR, resulting in an in frame fusion, and the ligated products were amplified using primers AcmAFsca and AcmAreveco. The resulting PCR products were subsequently digested with *Sca*I and *Eco*RI and cloned in the corresponding sites of plasmid pGKAL1, resulting in plasmids pGKAL8 and pGKAL9.

Overexpression and isolation of the AcmA active site domain

A DNA fragment encoding the active site domain of AcmA was obtained using the primers ACMHIS and REPDEL-3 with plasmid pAL01 as a template. The 504-bp PCR fragment was digested with *Bgl*II and *Eco*RI and subcloned into the *Bam*HI and *Eco*RI sites of pET32A (Novagen R&D Systems Europe Ltd, Abingdon, UK). The proper construct, pETAcmA, was obtained in *E. coli* BL21(DE3) [34]. Expression of the thioredoxin/AcmA fusion protein was induced in this strain by adding IPTG (to 1 mM final concentration) at $D_{600} = 0.7$. Four hours after induction the cells from 1 mL of culture were collected by centrifugation and the fusion protein was purified over a Talon™ metal-affinity resin (Clontech Laboratories, Palo Alto, CA) using 8 M urea-elution buffer and the protocol of the supplier. The eluate (200 µL) was dialyzed against a solution containing 50 mM NaCl and 20 mM Tris (pH 7) after which CaCl_2 was added to a final concentration of 2 mM. One unit of enterokinase (Novagen) was added and the mixture was incubated at room temperature for 20 h. The protein mixture was dialyzed against several changes of demineralized water before SDS/PAGE analysis. The thioredoxin part of the fusion protein was removed from the protein mixture with a Talon™ metal-affinity resin, as described above. Polyclonal antibodies were produced by Eurogentec (Ougrée, Belgium).

Binding of AcmA and its derivatives to lactococcal cells

The cells of 2 mL of exponential phase cultures of MG1363*acmAΔ1* were gently resuspended in an equal volume of supernatant of similarly grown NZ9000 (*acmAΔ1 ΔhtrA*) carrying either plasmid pGKAL1, -3, -4 or -7 and incubated at room temperature for 5 min. Subsequently, the mixtures were centrifuged. The supernatants were concentrated using phenol and ether extraction [35].

The pellets, containing cell-bound AcmA, were washed once with fresh $\text{G}^{1/2}\text{M17}$ medium and subsequently resuspended in 100 µL SDS/PAGE sample buffer [36]. Bound protein was released from the cell by boiling the samples for 5 min. The samples were subjected to SDS/12.5% PAGE followed by western hybridization analysis as described below.

SDS/PAGE and detection of AcmA activity

SDS/PAGE was carried out as described by Laemmli [36] with the protean II minigel system (Bio-Rad) and gels were stained with Coomassie Brilliant Blue (Bio-Rad). Standard low-range and prestained low- and high-range SDS/PAGE molecular mass markers of Bio-Rad were used as references.

AcmA activity was detected by a zymogram staining technique using SDS/PAGE (12.5%) gels containing 0.15% autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma) as described previously [3]. After electrophoresis, proteins were renatured using the AcmA renaturation solution [37].

Western blotting and immunodetection

Proteins were transferred from SDS/PAA (poly acrylamide) gels to BA85 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) or poly(vinylidene difluoride) membranes (Roche) as described previously [38]. AcmA antigen was detected with 5000-fold diluted rabbit polyclonal anti-AcmA IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG (Pharmacia) using the ECL detection system and protocol (Amersham).

Enzyme assays and absorbance measurements

AcmA activity was visualized on $\text{G}^{1/2}\text{M17}$ agar plates containing 0.2% autoclaved, lyophilized *M. lysodeikticus* cells as halos around the colonies after overnight growth at 30 °C. PepX was measured using the chromogenic substrate Ala-Pro-*p*-nitroanilid (BACHEM Feinchemicalien AG, Bubendorf, Switzerland), as described previously [4].

Immunofluorescence microscopy

Samples (25 µL) of *L. lactis* MG1363*acmAΔ1* or *Lb. casei* ATCC393 were pelleted and washed once with 1 mL of water. The pellets were resuspended in *L. lactis* NZ9000 (*acmAΔ1 ΔhtrA*) supernatant of overnight cultures containing the AcmA derivatives A1, A2, A3 or A4 and incubated for 5 min at room temperature. The cells were washed once with 1 mL of $\text{G}^{1/2}\text{M17}$ and subsequently incubated for 10 min at room temperature in 100 µL NaCl/ P_i (75 mM, pH 7.3, 68 mM NaCl) containing 4% bovine serum

albumin. Subsequently, the cells were resuspended in 100 μ L NaCl/P_i containing anti-AcmA IgG (diluted 1 : 400) with 2% bovine serum albumin for 1 h. After three washing steps with 1 mL of NaCl/P_i containing 0.1% Tween-20 (Sigma) (PBST), the cells were incubated for 1 h in 2% BSA in NaCl/P_i, containing a 1 : 400 dilution of Oregon Green anti-rabbit IgG (Molecular Probes, Eugene, OR). Subsequently, the cells were washed three times with 1 mL of PBST. The cells were transferred to a poly(L-lysine)-coated microscope slide (Omnilabo, Breda, the Netherlands), which was air-dried at ambient temperature. Vectashield (Vector, Burlingame, CA) was added, a cover slip was mounted and fluorescence was visualized with a Zeiss microscope (Carl Zeiss, Thornwood, NY) and an Axion Vision camera (Axion Technologies, Houston, TX).

Isolation of AcmA from the supernatant of *L. lactis* cultures

The cells of 100 mL of *L. lactis* MG1363*acmAA1* were boiled in 20 mL of 10% SDS for 15 min, washed six times with water and subsequently boiled in 20 mL of 10% (v/v) TCA and washed again six times with water. Subsequently, the cells were mixed with the supernatant of an overnight culture of *L. lactis* MG1363, containing AcmA. The mixture was stirred for 10 min at room temperature, centrifuged and the pellet was washed once with 50 mL of water. The pellet was resuspended in 10 mL 8 M LiCl and the pH was adjusted to 11.0 with NaOH. The suspension was incubated on ice for 15 min while vortexing every 3 min and subsequently centrifuged (15 min, 13 000 g). The supernatant, containing AcmA was dialyzed three times against 2 L of water and the dialyzed enzyme extract was stored at -20°C . Using the same method peptidoglycan-binding proteins were isolated from the supernatant of *L. lactis* MG1363*acmAA1*.

Determination of the hydrolytic activity of AcmA using RP-HPLC analysis

B. subtilis peptidoglycan was isolated as described previously [21]. Purified peptidoglycan (10 mg) was incubated with partially purified AcmA in 1 mL 50 mM KP_i buffer (pH 5.5) and incubated overnight at 37°C . As a control, the same amount of peptidoglycan was incubated with the peptidoglycan-binding protein preparation isolated from the supernatant of *L. lactis* MG1363*acmAA1*. The suspensions were centrifuged (15 min at 20 000 g) and the supernatants were split into pellet and supernatant fractions. To the pellets Cellosyl was added (250 $\mu\text{g}\cdot\text{mL}^{-1}$) and the suspensions were incubated overnight at 37°C and reduced with borohydride as described previously [21]. The supernatants were split in two: 0.5 mL was reduced with borohydride, and Cellosyl (250 $\mu\text{g}\cdot\text{mL}^{-1}$) was added to the other 0.5 mL, and

the suspension was incubated overnight at 37°C , after which the mixture was centrifuged and the supernatant reduced with borohydride. Reduced mucopeptide samples were separated using a Waters HPLC system and a Hypersil octadecylsilane column from Sigma (4.6×250 mm; particle size, 5 μm) as described previously [21,39]. Traces were spiked with purified mucopeptides [9] by addition to the reduced mucopeptide samples described above.

To determine whether AcmA could hydrolyze small, soluble peptidoglycan fragments, peptidoglycan fragments released from *B. subtilis* peptidoglycan by Cellosyl were incubated with AcmA as described above. The sample was reduced with borohydride as described above and the mucopeptides were resolved by RP-HPLC.

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